PB86-129285

# Hazard Evaluation Division
# Standard Evaluation Procedure
# Avian Single-Dose Oral LD50 Test

(U.S.) Environmental Protection Agency
Washington, DC

Jun 85

U.S. Department of Commerce
National Technical Information Service
NTIS

PB86-129285

EPA-540/9-85-007
June 1985

# HAZARD EVALUATION DIVISION

# STANDARD EVALUATION PROCEDURE

# AVIAN SINGLE-DOSE ORAL $LD_{50}$

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REPRODUCED BY
NATIONAL TECHNICAL
INFORMATION SERVICE
U.S. DEPARTMENT OF COMMERCE
SPRINGFIELD, VA. 22161

50272-101

| REPORT DOCUMENTATION PAGE | 1. REPORT NO. | 2. | 3. Recipient's Accession No. PB86 129285/AS |
|---|---|---|---|

**4. Title and Subtitle**
HAZARD EVALUATION DIVISION, STANDARD EVALUATION PROCEDURE
Avian Single-Dose Oral LD50 Test

**5. Report Date**
June 1985

**6.**

**7. Author(s)**
John Bascietto

**8. Performing Organization Rept. No.**
EPA-540/9-85-007

**9. Performing Organization Name and Address**
U.S. Environmental Protection Agency/OPP/HED/(TS-769C)
401 M Street SW
Washington, D.C. 20460

**10. Project/Task/Work Unit No.**

**11. Contract(C) or Grant(G) No.**
(C)
(G)

**12. Sponsoring Organization Name and Address**
Same as #9.

**13. Type of Report & Period Covered**

**14.**

**15. Supplementary Notes**
Supplement to Pesticide Assessment Guidelines Subdivision E - Hazard Evaluation: Wildlife and Aquatic Organisms (EPA-540/9-82-024 and NTIS Order Number PB83-153908), October 1982.

**16. Abstract (Limit: 200 words)**

The Standard Evaluation Procedure (SEP) for the Avian Single-Dose Oral LD50 Test is a guidance document primarily intended for Agency reviewers and the regulated industry who evaluate ecological effects data specified in 40 CFR Part 158.145. The SEP is also intended to provide information to the general public indicating how the Agency evaluates these types of studies. As such, it is designed to supplement Subdivision E of the Pesticide Assessment Guidelines: Hazard Evaluation - Wildlife and Aquatic Organisms. This SEP provides an Introduction, Materials and Methods, Reporting Requirements, Reviewer Evaluation, and Appendix of appropriate methods to guide the review and scientific evaluation of pesticide effects on birds.

The SEP for Avian Single-Dose Oral LD50 Test is only one of a number of SEP's published by the National Technical Information Service as a supplement to Subdivision E of the Pesticide Assessment Guidelines.

**17. Document Analysis a. Descriptors**

**b. Identifiers/Open-Ended Terms**

**c. COSATI Field/Group**

| 18. Availability Statement | 19. Security Class (This Report) | 21. No. of Pages |
|---|---|---|
| Unclassified and freely available. | Unclassified. | 15 |
| | 20. Security Class (This Page) Unclassified. | 22. Price |

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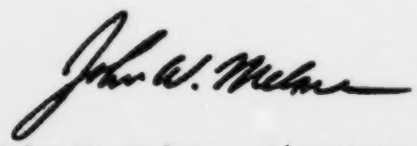
## STANDARD EVALUATION PROCEDURE

## PREAMBLE

This Standard Evaluation Procedure (SEP) is one of a set of guidance documents which explain the procedures used to evaluate environmental and human health effects data submitted to the Office of Pesticide Programs. The SEPs are designed to ensure comprehensive and consistent treatment of major scientific topics in these reviews and to provide interpretive policy guidance where appropriate. The Standard Evaluation Procedures will be used in conjunction with the appropriate Pesticide Assessment Guidelines and other Agency Guidelines. While the documents were developed to explain specifically the principles of scientific evaluation within the Office of Pesticide Programs, they may also be used by other offices in the Agency in the evaluation of studies and scientific data. The Standard Evaluation Procedures will also serve as valuable internal reference documents and will inform the public and regulated community of important considerations in the evaluation of test data for determining chemical hazards. I believe the SEPs will improve both the quality of science within EPA and, in conjunction with the Pesticide Assessment Guidelines, will lead to more effective use of both public and private resources.



John W. Melone, Director
Hazard Evaluation Division

# AVIAN SINGLE-DOSE ORAL LD50

## I. INTRODUCTION

### A. When Required

Avian single-dose oral toxicity tests are required to support registration of an end-use product intended for outdoor application, and to support registration of a manufacturing-use product which may be used to make such an end-use product. These studies are also required, on a case-by-case basis, to support the registration of an end-use product intended solely for indoor application, and to support registrations of manufacturing-use products which may be used to make such an end-use product.

### B. Purpose

Avian single-dose oral toxicity tests determine the median lethal dose ($LD_{50}$) of a chemical to birds. The $LD_{50}$ is defined as the quantity of toxicant calculated to kill fifty percent of the test population. These tests have attained broad acceptance among environmental toxicologists as relatively rapid, uncomplicated, inexpensive and statistically reliable methods for assessing short-term adverse effects of chemicals on avian wildlife.

The Ecological Effects Branch regularly requires that results of one avian acute test (one upland game bird or one waterfowl) be submitted to support the registration of a pesticide. The data from this test are used to:

- establish acute toxicity levels of the active ingredient to nontarget avian wildlife;
- compare toxicity information with measured or estimated amounts of pesticide residue in the terrestrial environment which may be consumed by birds in a single oral dose, in order to assess potential impact to avian wildlife;
- provide support for precautionary label statements to minimize adverse effects to avian wildife; and
- indicate the need for further testing and/or field studies.

### C. Test Substance

#### 1. Technical Grade

Routine tests must be conducted with the technical grade of the active ingredient (a.i.). If more than one a.i. constitutes a technical product then the technical grade of each a.i. must be

tested separately.

2. Formulated Product

The Pesticide Assessment Guidelines, Subdivision E, indicate when formulated products should be used in this test. Normally there will be a specific Agency requirement if such testing is called for.

## II. MATERIALS AND METHODS: TESTING STANDARDS/RECOMMENDATIONS

### A. Acceptable Protocols

Because the acute test is an established technique for assessing toxicity of a chemical to avian wildlife, much of the methodology for performing these studies, as well as the procedures for statistical analysis of results, have been carefully outlined and documented in the published literature. Notably, the information to be discussed in this Standard Evaluation Procedure (SEP) is presented in greater detail in the following reference:

Ecological Effects Branch. 1982. Pesticide Assessment Guidelines Subdivision E, Hazard Evaluation: Wildlife and Aquatic Organisms, EPA-540/9-82-024. pp. 33-37.

### B. Test Organisms

1. Acceptable Species/Age/Physical Condition

Testing must be done on either a wild waterfowl species, preferably mallard duck (Anas platyrhynchos), or an upland game species, preferably bobwhite quail (Colinus virginianus). The age of the test birds should be at least 16 weeks old when testing is initiated. The birds should be of uniform size and weight and be phenotypically indistinguishable from wild birds.

The animals should not have been artificially brought into a reproductive state (as can result from certain photoperiod exposures) and if this has occurred it must be so stated and the individuals' sexes identified. Marked weight loss, sickness, or mortality greater than 10% during an acclimation period are all signs that the affected lot should not be used.

These species were chosen because of their commercial availability, ecological significance, broad geographical distribution, commercial and recreational importance, ease of handling in the laboratory, and their past use in toxicity testing and known susceptibility to chemical exposure. Other species may be acceptable on a case-by-case basis; however, this is discouraged, unless

specifically required by the Agency. The test species must be verified by its scientific name.

2. Source/Acclimation

Within a given test all organisms must be from the same source (this includes laboratory and commercial stocks) and most preferably from the same hatch. The birds should be preconditioned to test facilities for as long as possible (at least 15 days) prior to the beginning of the test.

C. Test Conditions

1. Number of Birds Per Level

The actual dose-levels for the "definitive" study should be determined by "range finding" studies. Results from "range finding" studies may not, however, be used to calculate the $LD_{50}$. Except in cases where the $LD_{50}$ is demonstrated to be greater than 2000 mg/kg, definitive studies must use a minimum of five treatment levels plus additional groups for control or vehicle control if a vehicle is used. Ten birds per treatment group and ten birds for each control and vehicle control group are strongly recommended. Concurrent control and vehicle control groups are required for each $LD_{50}$ test. In all cases the birds must be randomly assigned to pens.

Factors between dose-levels chosen for definitive studies should be based on a geometric scale and, using results from range finders, should attempt to produce mortality ranging from 10 to 90 percent. In order to provide statistically reliable results the design of the definitive study should attempt to produce at least three partial kills (i.e., between 0 and 100 percent) surrounding the estimated $LD_{50}$.

Studies should be designed to establish an actual $LD_{50}$ and 95% c.i.. In lieu of this a study may demonstrate that the $LD_{50}$ is greater than 2000 mg/kg. In this case the study should show, on the basis of ten or more birds per dose, that less than one-half of the birds died at 2000 mg/kg. When any mortality is observed at 2000 mg/kg, sequentially lower levels must be tested (with ten birds per level) in order to get a dose-response series which includes at least one "no-effect" (no mortality) level.

2. Pen Facilities

Pen conditions should conform to good husbandry practices, and must be large enough to accommodate the birds. Cages should not create crowding stress, because this could result in aggressive or self-destructive behavior.

3. Photoperiod

The Agency now recognizes that the variety of lighting regimes allowed by the current $LD_{50}$ guideline could create a problem of artificial inductance of reproductive states in 16 week old birds if too much light is provided. While the guideline will be revised as soon as possible, investigators may avoid this problem by providing a 10-hour light/14-hour dark lighting regime (flourescent or incandescent sources).

4. Body Weights

Individual body weights should be measured at the beginning and the end of the study.

5. Food Consumption and Diet

Feed should be withheld from all birds for at least 15 hours prior to oral dosing. After administration of the test material the birds should have free access to feed (a standard game bird ration) and water. Food consumption must be monitored for treated and control birds, but can be calculated as average daily food consumption at each dose-level.

6. Dose Preparation/Administration

The test material should be administered without a "vehicle" if possible. If an evaporative vehicle is used (such as acetone or methylene chloride) in preparing the material for use with capsules, it should be completely evaporated at room temperature prior to placing in capsules. The material should be accurately weighed then placed in gelatin capsules. Acceptable vehicles include corn oil, propylene glycol, 1% carboxymethylcellulose and gum arabic. Maximum vehicle amount per dose should not exceed 0.1 to 1.0% of body weight, and should be used on a constant volume/body weight basis. Vehicle control groups must receive the maximum amount of vehicle available to any treatment group. Water should be freely available throughout the test.

7. Observation Period

Except in cases where no birds survive, the observation period is always a minimum of 14 days after dosing. Control mortality must not exceed ten percent during the same observation period used for the experimental animals. The study must be extended beyond 14 days if toxic signs persist or birds continue to die on the last day (Day 14) of the observation period.

## III. REPORTING REQUIREMENTS

### A. Test Material

The purity of the test material must be stated and should routinely be "technical" grade unless otherwise required by the Agency. The percent active ingredient in all test materials must be stated.

### B. Mortality/Observable Effects Criteria

The criteria for determining effects must be defined. The raw data or percentage of deaths/effects at each test level and control group as well as the number of birds tested per group must be reported.

### C. Calculated LD50

The statistically calculated $LD_{50}$ with 95% confidence interval and the method of calculation must be presented. In lieu of a calculated $LD_{50}$, the study may show that the $LD_{50}$ is greater than 2000 mg/kg. The slope of the dose-response line should be calculated and reported. Some reports have a graphic (log probit/dose level) presentation which is helpful.

### D. Body Weight and Food Consumption

The individual body weights and mean food consumption should be reported at the beginning of the test and on Day 14. If the test is extended beyond 14 days these measurements should be reported at the end of the study as well.

### E. Raw Mortality Data

Raw mortality data or percentage of deaths/effects must be reported. The data must indicate numbers of birds dying at each dose or control level. The day of death or effect must be reported; it is preferable to also report the time of death.

### F. Gross Necropsy

Gross necropsies are preferred. When performed, all dead birds should be examined, as well as a sufficient number of survivors in order to provide a characterization of gross lesions. Inspections of the GI tract, liver, kidneys, heart, and spleen should be made.

### G. Other Observations

Signs of intoxication should be described as to what was observed when and for how long. Technical terminology used to name signs of intoxication should be adequately defined if these

terms are not in general use. The report should indicate whether deaths are associated with specific signs of intoxication, and the time of onset and remission of toxic signs. If affected birds recover, the time interval to recovery should be reported. Marked observations of fecal material and urine should be reported.

H. Statistical Analysis

A statistical analysis of the mortality data is required. The $LD_{50}$, 95% confidence intervals, and slope of the dose-response line should be calculated using one of the methods referenced in the Pesticide Assessment Guidelines, Subdivision E. Other statistically valid methods such as computer software are acceptable subject to validation by the Agency. When reporting the $LD_{50}$ and 95% c.i., the slope of the dose-response line should be given (the actual dose-response plot need not be included).

I. Acceptable Protocols

EEB does not endorse any one protocol. It is sometimes necessary and desirable to alter the procedures presented in published protocols to meet the needs of the chemical or test organisms used. However, EEB does recommend some protocols as guidance for developing avian acute toxicity tests. These protocols include:

Ecological Effects Branch. 1982. Pesticide Assessment Guidelines Subdivision E, Hazard Evaluation: Wildlife and Aquatic Organisms, EPA-540/9-82-024. pp. 33-37.

This referenced protocol is presented as flexible guidance to help researchers design scientific protocol and to help the reviewer validate studies. It is important to recognize that avian acute tests are validated based on whether they fulfill guideline requirements and whether they provide scientifically sound information on the acute toxicity of the test material to avian wildlife that is useful in risk assessments. This is more important than whether they follow a referenced protocol step-by-step.

IV. REVIEWER'S EVALUATION

The reviewer should identify each aspect of the reported procedure that is inconsistent with recommended protocol. The significance of these deviations must be determined. The number of deviations and their severity will determine the validity of the study and the interpretation of the results.

A. Review of Materials and Methods

1. Test Substance

When the technical grade is greater than 80% a.i., the reviewer may elect not to adjust the definitive $LD_{50}$ value to reflect actual amount of a.i.. Reviewers should check the accuracy of the reported $LD_{50}$ value by recalculating the doses in mg/kg a.i..

2. Diet

A review of the diet composition should be made specifically to check for unfamiliar ingredients or medications. Any such unfamiliar ingredient or suspected additive should be verified by contacting the testing laboratory prior to classifying the study.

3. Body Weight and Food Consumption

Vomiting is an indication that the dose is rejected. If this is a problem, the test may need to be rerun. Bobwhite quail are generally less prone to dose rejection than mallards. The vehicle or additive may have to be changed or removed. Perhaps passerines or other birds could be tested to get around this problem.

Body weight and food consumption data can be used to evaluate effects, if any, on feeding and/or weight change.

4. Mortality Data

Raw mortality data or percentage of deaths/effects must be checked to insure consistency with the written report. In cases where the two do not agree, the discrepancies must be explained in writing prior to classifying the study.

The control mortality data must be checked. Generally, not more than 10% control mortality will be acceptable.

B. Verification of Statistical Analyses

The reviewer should "validate" the statistical analyses of the data. This may be done using EEB's "Toxanal," "SAS" or other computer programs which calculate the $LD_{50}$, 95% c.i., and the slope.

The statistics presented in the report should be verified particularly if the raw data do not show clear linear dose/mortality response or if the reported $LD_{50}$ and confidence limits do not seem to match the raw data. Any deviation should be noted and explained if possible.

Adjustments to the $LD_{50}$ may be made at this time to reflect the reviewer's estimate of the $LD_{50}$. These may be necessary in cases where the test material a.i. content was low or to correct errors in the authors' calculations.

C. Discussion of Results

1. Mortality and Behavioral Observations

The reviewer should discuss the results in light of the observations of intoxication. Mortality which cannot be fully attributed to the effects of the test material may be better understood if assessed in light of behavioral observations. Also, signs of intoxication could aid in understanding potential sublethal effects which could effect the birds' ability to develop and survive in the wild.

2. Implications of Dose/Mortality Response

The dose/mortality response enables derivation of such useful information as the $LD_{50}$ and 95% confidence interval as well as the observed NOEL. It may also reveal important characteristics about the toxicity of the test material, such as whether the response is gradual over a wide range of test levels or rapid with a narrow range between the observed NOEL and 100% mortality.

Pertinent data on dose/mortality response should be included in data evaluation records.

3. Gross Necropsy

The results of gross necropsies, when performed, may be helpful in evaluating the study. When the same lesions are found in dead and surviving experimental birds, this may be an indication that a longer observation period may be necessary. If lesions are found in reproductive organs, reproduction studies may be indicated. Lesions found in dead controls are an indication that the lot should not have been used.

4. General Comments

Reviewers should comment on the protocol used as well as the methods or other aspects of the study which are irregular, unfamiliar, or unacceptable. Suggestions for improvements to the protocol can be made, as well as giving rationale for rejecting certain methods or aspects of the study. Of particular importance are the reviewer's comments on any aspect of the study, such as condition of the birds, husbandry practices, dose administration or rejection, toxic signs, or environmental conditions, which could bear on the interpretation of the results.

D. Descriptive Categorization of Results

The reviewer should indicate what the results were and how much information can be drawn from them. At a minimum, an avian single-dose oral toxicity test will provide an $LD_{50}$ with 95% confidence intervals. This should allow classifying the test material based on the following scheme:

| $LD_{50}$ (mg/kg) | Category Description |
|---|---|
| < 10 | very highly toxic |
| 10 - 50 | highly toxic |
| 51 - 500 | moderately toxic |
| 501 - 2000 | slightly toxic |
| > 2000 | practically non-toxic |

These descriptive categories are somewhat arbitrary and are for in-house use only; mainly for inter-chemical comparison. These categories do not necessarily reflect actual environmental hazard to avian wildlife, nor is the descriptive verbiage used in determining risk. The latter task ultimately rests on quantitative toxicity and exposure data.

E. Reviewer's Conclusions

1. Category

The reviewer should indicate under which of the three validation categories the study fits:

° Core: All essential information was reported and the study was performed according to recommended protocols. Minor inconsistencies with standard methodologies may be apparent; however, the deviations do not detract from the study's soundness or intent. Studies within this category fulfill the basic requirements of current guidelines and are acceptable for use in a risk assessment.

° Supplemental: Studies in this category are scientifically sound; however, they were performed under conditions that deviated substantially from recommended protocols. Results do not meet guideline requirements; however, the information may be useful in a risk assessment. Some of the conditions that may place a study in a supplemental category include, but are not limited to:

- Unacceptable test species;
- Inappropriate test material; or
- Dosage levels tested were less than 2000 mg/kg but not high enough to produce an effect on the organisms or a precise $LD_{50}$.

- Invalid: These studies provide no useful information. They may not be scientifically sound, or they were performed under conditions that deviated so significantly from recommended protocols that the results will not be useful in a risk assessment.

  A study where the test material was not properly identified can be invalidated.

2. Rationale

Identify what makes the study supplemental or invalid. It may also be necessary to justify a higher category in spite of deviations. That is, a study may have been called core or supplemental even though there were substantial deviations from recommended protocol. While all deviations should be noted, it may be that the deviations did not actually alter the response of the test organism to the test material. The reviewer is expected to exercise judgment in this area.

3. Repairability

Indicate whether the study may be upgraded or given a higher validation category if certain conditions are met. Usually this would involve the registrant submitting more data on the study.

F. References

The reviewer should reference any information used in the validation procedure. This should include protocol documents, statistical methods, or information taken from files of other divisions or branches within HED.